

CHARACTERIZATION OF GLUCOSE-6-PHOSPHATASE IN HEPATOCYTES

EFFECTS OF AMILORIDE AND PENTAMIDINE

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Abstract—The hepatic microsomal glucose-6-phosphatase enzyme is situated inside the lumen of the endoplasmic reticulum and, for normal enzyme activity *in vivo*, three transport systems are needed for the substrate glucose-6-phosphate and the products phosphate and glucose. Previous studies using isolated microsomes showed that the drugs amiloride and pentamidine do not affect the glucose-6-phosphatase enzyme but can activate the glucose-6-phosphate transport system. Here we demonstrate that, very surprisingly, the addition of pentamidine (and to a lesser extent amiloride) to isolated hepatocytes results in an inhibition of the catalytic subunit of glucose-6-phosphatase.

Glucose is the primary energy source for virtually all tissues. The maintenance of blood glucose levels within a narrow range is, therefore, essential for the normal metabolic functioning of many tissues including brain. At times of stress or whenever blood glucose levels start to fall, the liver releases glucose into the bloodstream to maintain blood glucose homeostasis. Glucose-6-phosphatase (EC 3.1.3.9) catalyses the terminal step of the two pathways by which the liver makes glucose, namely gluconeogenesis and glycogenolysis [1, 2]. Any drug which affects glucose-6-phosphatase activity, therefore, has the potential to alter the hepatic output of glucose. Unlike all the other enzymes of gluconeogenesis and glycogenolysis, the catalytic subunit of the glucose-6-phosphatase enzyme is situated with its active site inside the lumen of the endoplasmic reticulum [3, 4]. It is, therefore, necessary to have the means to transport the substrates and products of the enzyme across the endoplasmic reticulum membrane (see Fig. 1). There are three transport systems associated with the glucose-6-phosphatase enzyme termed T₁, T₂ and T₃ (or GLUT 7) which transport glucose-6-phosphate, phosphate (and pyrophosphate) and glucose, respectively, across the endoplasmic reticulum membrane [5–7]. Although two of the transport proteins have now been purified [8, 9], very little is known about the regulation of any of the transport proteins.

Recently, we demonstrated that the drugs amiloride and pentamidine (see Fig. 2) both activate T₁ (by reducing its K_m) when added to isolated microsomes *in vitro* [10, 11]. We have, therefore, studied the effects of the addition of amiloride and pentamidine to hepatocytes to determine the effects of the drugs under more physiological conditions.

Surprisingly, the predominant effect of the addition of amiloride or pentamidine to hepatocytes is not an activation of T₁ but inhibition of the glucose-6-phosphatase enzyme.

MATERIALS AND METHODS

Materials. Glucose-6-phosphate (monosodium salt), sodium dodecyl sulphate (especially purified for biochemical work) and tetrasodium pyrophosphate were purchased from BDH (Poole, U.K.). Mannose-6-phosphate (disodium salt), amiloride hydrochloride [3,5-diamine-6-chloro-*N*-(diaminomethylene pyrazine carboxamide)] (see Fig. 2), pentamidine [1,5-is (*p*-aminophenoxy)-pentane bis (2-hydroxyethanesulfonate isethionate salt)] (see Fig. 2) and streptozotocin were from the Sigma Chemical Co. (Poole, U.K.). Cacodylic acid also from Sigma was recrystallized from 95% ethanol [12]. All other chemicals were analytical grade reagents.

Hepatocyte isolation and culture. Adult male Wistar rats (200–250 g) were used throughout. Animals were allowed free access to food and water (fed) or were denied food but not water for 16 hr prior to killing (starved), or had diabetes induced by a single tail vein injection of streptozotocin (75 mg/kg body wt in buffered citrate, pH 4.5) [13]. Hepatocytes were isolated by collagenase perfusion of the liver [14]. Cell viability estimated by Trypan blue exclusion was >90% and the cell yield was routinely about 50% of the initial liver weight. The final pellet was resuspended in Krebs–Henseleit buffer minus phosphate to give routinely approximately 5 mg dry weight of cells per mL. For perfusion of livers from fed and diabetic rats, all Krebs–Henseleit buffers contained 5 mM D-glucose to minimize glycogen depletion.

Cell suspensions were incubated for 20 min at 37°, prior to addition of any drug, in a shaking water bath (100 cycles/min) under continuous gassing (95% O₂, 5% CO₂). Aliquots were taken and 1 mM

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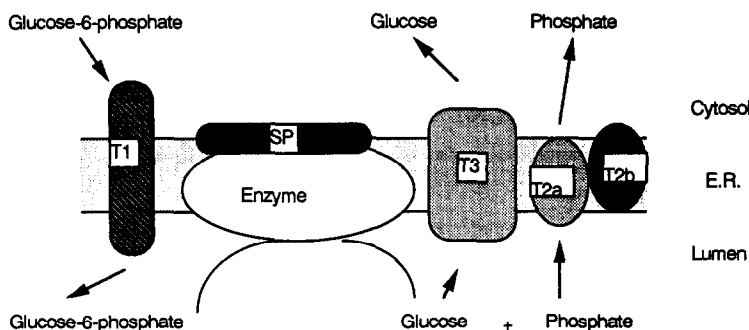


Fig. 1. A schematic representation of hepatic microsomal glucose-6-phosphatase. T1, glucose-6-phosphate transport protein; T2 a and b, phosphate and pyrophosphate transport; T3, GLUT 7, the microsome glucose transport protein; SP, a regulatory protein termed stabilizing protein; E.R., endoplasmic reticulum.

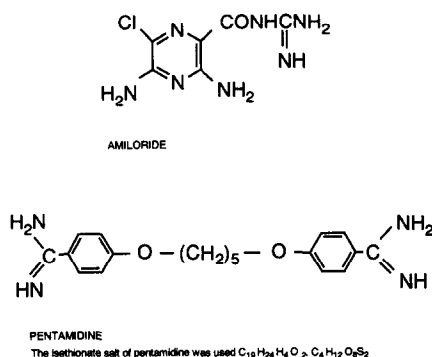


Fig. 2. The structure of amiloride and pentamidine.

amiloride or 1 mM pentamidine added as indicated. At time 0 and 30 min after addition of drug, aliquots of the control and test cell suspensions were analysed.

Preparation of hepatocytes for glucose-6-phosphatase assay. All buffers were prepared minus phosphate. Aliquots of cells were homogenized in a Jencons glass homogenizer (30 strokes) and spun for 15 sec at 1000 g. The 1000 g supernatants were assayed immediately.

Preparation of liver homogenates and microsomes. Livers were homogenized in 9 parts (w/v) of ice-cold 0.25 M sucrose, 5 mM Hepes buffer pH 7.4 in a Teflon-glass homogenizer. A proportion of the resulting 10% homogenate was then used to prepare microsomes as described previously [15].

Assays. Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed and calculated as described by Burchell *et al.* [16] and expressed as nmol P_i released/min/mg of protein. All assays were linear with respect to incubation time. The concentrations of the substrate glucose-6-phosphate used to calculate the kinetic constants shown in Tables 1, 2 and 3 were 1, 1.4, 2, 2.6, 5 and 30 mM. Kinetic constants, V_{\max} and K_m , were calculated using a BBC computer programme of non-linear multiple regression analysis based on that of Colquhoun [17]. The microsomes present in 1000 g supernatant or

the 105,000 g microsomal pellet are always a mixture of intact and disrupted structures. The proportion of intact microsomes was determined in all preparations by assays of low K_m (1 mM) mannose-6-phosphatase activity which is only expressed in disrupted structures [18]. All of the preparations used in this paper were more than 90% intact in both the absence and presence of the drugs used. The intact values reported in Tables 1–4 have been corrected to allow for the percentage of disrupted microsomal structures present in each preparation, eliminating the large errors in activity measurements that can occur if even a small proportion of the microsomal vesicles are disrupted. The concentrations of pentamidine and amiloride used where indicated in Tables 3 and 4 were 50 μ M and 5 mM, respectively, which were shown previously to be optimal concentrations for addition to microsomes without loss of microsomal intactness [10, 11]. Protein concentrations were estimated by the method of Lowry as modified by Peterson [19]. Statistical analysis was performed using Student's *t*-test for paired samples as described previously [20].

RESULTS

The glucose-6-phosphatase activity in intact microsomal structures is a measure of the combined rates of the glucose-6-phosphatase enzyme and the three transport systems T₁, T₂ and T₃ which transport glucose-6-phosphate, phosphate and glucose, respectively, across the microsomal membrane (see Fig. 1). The addition of pentamidine to hepatocytes isolated from fed, starved and diabetic rat livers caused a significant decrease in the V_{\max} of the glucose-6-phosphatase system without significantly altering the K_m which only decreased by a small amount (Table 1). Addition of amiloride caused a smaller decrease in the V_{\max} of the glucose-6-phosphatase system which was only significant in fed hepatocytes (Table 1) and no significant change in the K_m . Figure 3 is a Hanes plot showing the data obtained in one of the sets of experiments shown in Table 1.

To determine whether pentamidine (and to a

Table 1. Effects of amiloride and pentamidine on intact microsomal glucose-6-phosphatase system activity in hepatocytes

Incubation time (min)	0		30	
Glucose-6-phosphatase system activity	V_{\max} (nmol/min/mg)	K_m (mM)	V_{\max} (nmol/min/mg)	K_m (mM)
Fed				
Control	51 ± 10	4.0 ± 0.8	40 ± 4	2.6 ± 0.7
Amiloride	28 ± 3*	3.0 ± 0.6	37 ± 6	3.0 ± 0.4
Pentamidine	30 ± 10*	1.8 ± 0.1	25 ± 1*	2.0 ± 0.4
Starved				
Control	52 ± 5	2.6 ± 0.4	60 ± 3	2.5 ± 0.5
Amiloride	48 ± 5	2.4 ± 0.3	59 ± 7	3.0 ± 0.2
Pentamidine	31 ± 8*	2.1 ± 0.3	23 ± 3†	1.6 ± 0.3
Diabetic				
Control	65 ± 6	2.9 ± 0.4	66 ± 3	3.0 ± 0.6
Amiloride	44 ± 10	1.8 ± 0.4	51 ± 20	3.0 ± 0.9
Pentamidine	36 ± 6†	1.6 ± 0.2	37 ± 6*	2.1 ± 0.5

Data are the means ± SEM from three or four separate preparations. Differences calculated from control values. *P < 0.05, †P < 0.01.

Table 2. Effects of amiloride and pentamidine on glucose-6-phosphatase catalytic subunit activity in hepatocytes

Incubation time (min)	0		30	
Glucose-6-phosphatase system activity	V_{\max} (nmol/min/mg)	K_m (mM)	V_{\max} (nmol/min/mg)	K_m (mM)
Fed				
Control	62 ± 10	1.5 ± 0.3	50 ± 4	1.4 ± 0.1
Amiloride	36 ± 6	1.1 ± 0.1	44 ± 6	2.0 ± 0.1
Pentamidine	38 ± 10	1.5 ± 0.5	29 ± 2*	1.9 ± 0.6
Starved				
Control	68 ± 7	1.3 ± 0.3	67 ± 3	1.3 ± 0.2
Amiloride	66 ± 8	1.4 ± 0.3	68 ± 5	2.0 ± 0.3
Pentamidine	46 ± 5*	1.4 ± 0.1	28 ± 4†	1.4 ± 0.2
Diabetic				
Control	69 ± 4	1.8 ± 0.4	74 ± 10	1.7 ± 0.2
Amiloride	57 ± 10	1.5 ± 0.3	52 ± 2*	1.7 ± 0.5
Pentamidine	43 ± 6*	1.0 ± 0.2	37 ± 6*	1.1 ± 0.1

Data are the means ± SEM from three or four separate preparations. Differences calculated from control values. *P < 0.05, †P < 0.01.

Table 3. Effects of amiloride and pentamidine on glucose-6-phosphatase activity in liver microsomes

	Intact microsomes		Disrupted microsomes	
	V_{\max} (nmol/min/mg)	K_m (mM)	V_{\max} (nmol/min/mg)	K_m (mM)
Fed				
Control	226 ± 40	3.1 ± 1.2	259 ± 10	0.9 ± 0.04
Amiloride	184 ± 20	1.0 ± 0.9*	219 ± 10	0.7 ± 0.2
Pentamidine	171 ± 10	1.1 ± 0.3†	238 ± 6	0.6 ± 0.1
Starved				
Control	385 ± 70	3.5 ± 0.3	553 ± 130	1.0 ± 0.4
Amiloride	535 ± 6	2.3 ± 0.7	579 ± 30	1.6 ± 0.4
Pentamidine	516 ± 9	1.7 ± 0.3†	574 ± 60	0.9 ± 0.1
Diabetic				
Control	576 ± 60	4.2 ± 1.1	1178 ± 90	1.2 ± 0.1
Amiloride	636 ± 103	1.6 ± 0.4*	977 ± 67	0.6 ± 0.1*
Pentamidine	477 ± 200	1.1 ± 0.1†	957 ± 130	0.7 ± 0.04

Data are means ± SEM from three separate preparations. Differences calculated from control values. *P < 0.07, †P ≤ 0.05.

Table 4. Effects of amiloride and pentamidine on glucose-6-phosphatase activity in liver homogenates

	Intact microsomes		Disrupted microsomes	
	V_{\max} (nmol/min/mg)	K_m (mM)	V_{\max} (nmol/min/mg)	K_m (mM)
Fed				
Control	42 ± 2	3.2 ± 0.2	49 ± 2	1.3 ± 0.2
Amiloride	34 ± 1	1.5 ± 0.3*	36 ± 4	1.0 ± 0.2
Pentamidine	35 ± 3	1.4 ± 0.4*	36 ± 4	0.9 ± 0.4
Starved				
Control	42 ± 5	2.5 ± 0.9	45 ± 20	0.7 ± 0.3
Amiloride	46 ± 9	1.4 ± 0.3	65 ± 6	0.7 ± 0.1
Pentamidine	61 ± 2	1.8 ± 0.6	79 ± 4	0.6 ± 0.2
Diabetic				
Control	99 ± 2	4.8 ± 0.7	129 ± 30	0.8 ± 0.2
Amiloride	78 ± 4	1.7 ± 0.3†	109 ± 70	0.9 ± 0.4
Pentamidine	75 ± 5*	2.0 ± 0.6†	129 ± 6	0.9 ± 3

Data are means ± SEM from three separate preparations. Differences calculated from control values. *P < 0.07, †P ≤ 0.05.

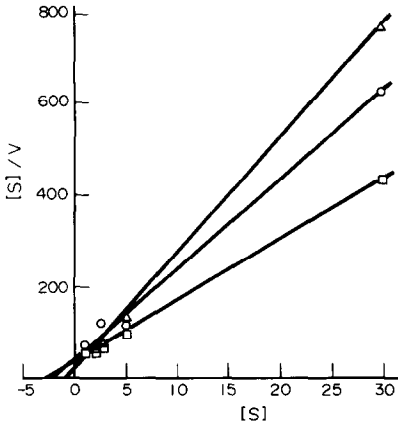


Fig. 3. Hanes-Woolf plot of amiloride and pentamidine inhibition of glucose-6-phosphatase system activity in diabetic rat hepatocytes. (□) Control; (○) amiloride; (△) pentamidine.

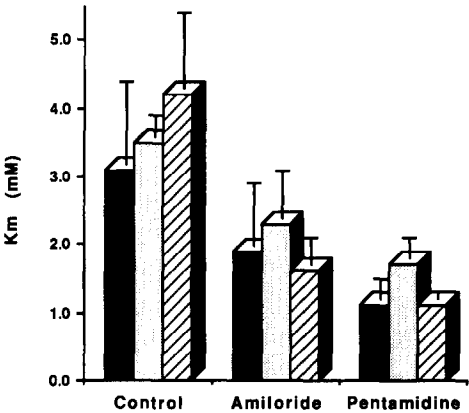


Fig. 4. Effect of amiloride and pentamidine on the K_m of fed, starved and diabetic intact rat liver microsomes. (■) Fed; (▨) starved; (□) diabetic. Amiloride 5 mM, pentamidine 50 μ M.

lesser extent amiloride) was inhibiting the glucose-6-phosphatase enzyme or one of the transport proteins, the activity of the glucose-6-phosphatase enzyme was measured after the microsomal vesicles had been fully disrupted. Pentamidine decreased the V_{\max} of the glucose-6-phosphatase enzyme in hepatocytes isolated from fed, starved and diabetic rats (Table 2). Amiloride caused a smaller decrease in the V_{\max} of the glucose-6-phosphatase enzyme which was only significant in hepatocytes isolated from diabetic rats. In all cases, the effects of amiloride and pentamidine were similar (although not always significant) if drug was added to the hepatocytes immediately before homogenization of the cells (0 time), or if the hepatocytes were incubated for 30 min in the presence of drug prior to homogenization (30 min) (Tables 1 and 2). The assays of glucose-6-phosphatase activity in hepatocytes shown in Tables 1 and 2 were all carried

out using homogenates of hepatocytes. To determine whether there are any differences in the effects of pentamidine and amiloride in homogenates and microsomes, the drugs were added directly to liver microsomes (Table 3 and Fig. 4) and liver homogenates (Table 4). In both cases, drug had no significant effect on the activity of the glucose-6-phosphatase enzyme measured in fully disrupted microsomes. In contrast they lowered significantly the K_m of glucose-6-phosphatase activity in intact microsomes (Table 3, Table 4 and Fig. 4).

DISCUSSION

In order to allow interpretation of the effects of the administration of drugs to hepatocytes, it was first necessary to establish whether the activity of the glucose-6-phosphatase system in hepatocytes is the same as in isolated microsomes.

The activity (V_{\max}) of the catalytic subunit of the hepatic glucose-6-phosphatase enzyme is well known to vary in isolated microsomes depending on metabolic state. The activity of the enzyme (in control disrupted microsomes) was approximately twice as high in microsomes isolated from starved rat livers as in fed rat liver microsomes and the activity approximately doubled again in diabetic liver microsomes (Table 3). This is very similar to results found many times previously (e.g. Refs 2, 3, 10, 11, 13, 21, 22). Similar results were also obtained in liver homogenates (Table 4). It was, therefore, surprising to find that the activity of the catalytic subunit of the glucose-6-phosphatase enzyme in control diabetic hepatocytes was only approximately 50% higher than in control fed hepatocytes (Table 2) rather than the 300–400% increase observed in diabetic microsomes and diabetic liver homogenates (Tables 3 and 4). Immunoblot analysis with antibody found previously to be monospecific for the catalytic subunit of the glucose-6-phosphatase enzyme [23, 24] revealed that the hepatocytes contained glucose-6-phosphatase catalytic subunit of normal molecular weight (data not shown). The simplest explanation is, therefore, that the activity of the glucose-6-phosphatase enzyme is modified during the preparation of hepatocytes, for example by covalent modification. Another difference between the glucose-6-phosphatase enzyme in hepatocytes and fresh liver samples is that its K_m is up to twice as high in hepatocytes (Table 2) as in liver (Tables 3 and 4).

There have been many reports of inhibition of glucose-6-phosphatase activity in microsomes (see Ref. 1 for a review) but very few reports of activation of any of the individual protein components of glucose-6-phosphatase. Two drugs amiloride and pentamidine were recently shown to activate T_1 (see Fig. 1) in isolated microsomal preparations [10, 11]. Amiloride does not affect blood glucose homeostasis in man, presumably because amiloride does not cross cell membranes [25]. In contrast, pentamidine which has been used to treat trypanosomiasis and leishmaniasis for 40 years has dramatic effects on blood glucose homeostasis [26–30], and is known to accumulate in liver and kidney [28] which are the major sites of glucose-6-phosphatase in mammals [1, 2, 5–7]. *In vivo*, logically, amiloride would not be expected to affect glucose-6-phosphatase activity whereas, in contrast, it seemed probable that glucose-6-phosphatase activity would be activated by pentamidine administration *in vivo*. It was, therefore, unexpected to find that administration of pentamidine (and to a much lesser extent, amiloride) to hepatocytes resulted in the inhibition of the activity of the glucose-6-phosphatase enzyme (Tables 1 and 2). Pentamidine inhibited the glucose-6-phosphatase enzyme similarly whether the hepatocytes were homogenized immediately after administration or following 30 min incubation in the presence of the drug (Tables 1 and 2), ruling out the possibility that a metabolite of pentamidine produced in the hepatocytes is the inhibitor of the glucose-6-phosphatase enzyme.

One obvious difference between this and previous

studies was that in this study the glucose-6-phosphatase assays were carried out using homogenates of hepatocytes not isolated microsomes. However the administration of pentamidine or amiloride to glucose-6-phosphatase enzyme activity in liver homogenates did not result in a significant inhibition of glucose-6-phosphatase enzyme activity (Table 4). In Tables 3 and 4, 50 μ M pentamidine and 5 mM amiloride were used but previous studies [10, 11] have shown that 1 mM concentrations of both drugs also do not affect the activity of the glucose-6-phosphatase enzyme in isolated microsomes. The different effects of amiloride and pentamidine in hepatocytes and isolated microsomes cannot be due to the different concentrations of drug that were used in the experiments.

The administration of amiloride to hepatocytes has been shown previously to alter the activity of protein kinases and phosphatases [31, 32], and although there have been no studies on the effects of administration of pentamidine to hepatocytes on the phosphorylation status of proteins, this raises the possibility that the effects of amiloride and pentamidine in hepatocytes might be due to changes in the phosphorylation state of the glucose-6-phosphatase enzyme.

The results obtained demonstrate that measurements of the effects of adding drugs to microsomes on glucose-6-phosphatase activity cannot be used to predict the effects of the drugs on glucose-6-phosphatase activity *in vivo*. The hepatocyte studies suggest that some of the many reported effects of pentamidine on blood glucose homeostasis [26–30] could be in part mediated by its action on the glucose-6-phosphatase system.

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